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Chemotaxonomy and Palynology of the *Citrus* Species in Iraq: A Biosystematics Study

Khulood Khalaf Jabbar and Arman Mahmoudi Otaghvari*

Department of Plant Science, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran

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****Corresponding authors:***
✉ A. Mahmoudi Otaghvari
botany1347@gmail.com

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ABSTRACT

The Rutaceae family plays an important role in the biodiversity of the ecosystem, as it comprises evergreen species that serves economic, health, and cosmetic purposes, among others. The aim of this study was to conduct a biosystematic investigation of the genus *Citrus* (Rutaceae) in Iraq using chemotaxonomic and palynological approaches. For this purpose, leaves of eleven *Citrus* species were collected from Diyala Province, Iraq. Methanolic leaf extracts were analyzed using gas chromatography-mass spectrometry (GC-MS), and pollen morphology was examined using field-emission scanning electron microscopy (FESEM). GC-MS spectrum analysis of the methanolic extracts of the eleven leaf extracts revealed qualitative and quantitative variation in the detected chemical compounds among the studied species. Hierarchical cluster analysis (HCA) based on the GC–MS data separated the species into two main clusters. The first cluster (C1) includes nine species, *C. sinensis*, *C. reshni*, *C. aurantium*, *C. latifolia*, *C. sinensis var.* Moro, *C. deliciosa*, *C. grandis*, *C. aurantiifolia var. acidica* and *C. paradisi*. The second cluster (C2) included *C. limetta* and *C. limon*. Palynological observations showed that the pollen grains of all examined species were unicellular, radially symmetrical, and isopolar. The grains were generally medium-sized, and their basic shapes included spheroidal to sub-prolate forms. Minor size variation was observed among species, but no major structural deviations were recorded. Overall, the chemotaxonomic and palynological data were congruent and provided complementary evidence for species delimitation within *Citrus*. These findings highlight the usefulness of integrating chemical profiling and pollen morphology in biosystematic studies of this genus.

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Introduction

The genus *Citrus* L., belonging to the family Rutaceae, comprises some of the most economically, nutritionally, and agronomically essential fruit crops cultivated worldwide, including in Iraq (Benedetto *et al*., 2023). Iraq, particularly regions with favorable subtropical microclimates such as Diyala province, has a long tradition of *Citrus* cultivation, hosting a wide array of species, cultivars, and local varieties. Despite its economic prominence, the systematic classification and species delimitation within the genus *Citrus* remain among the most complex and contentious topics in plant taxonomy (Zhao *et al*., 2025). This complexity primarily stems from extensive interspecific hybridization, a high frequency of somatic mutations, polyploidy, facultative apomixis (nucellar embryony), and centuries of human selection and germplasm exchange. Consequently, conventional macro-morphological traits-such as leaf shape, fruit size, rind color, and tree habit-exhibit considerable phenotypic plasticity and often overlap significantly among species and cultivars, rendering traditional morphological keys insufficient for unambiguous taxonomic identification.

To overcome the limitations of macro-morphology, modern biosystematics increasingly relies on integrative approaches that incorporate biochemical and micro-morphological markers. Chemotaxonomy has emerged as a powerful diagnostic tool, leveraging the structural diversity of secondary metabolites to infer systematic and evolutionary relationships among plant taxa. The chemical constituents of Citrus leaves—including essential oils, monoterpenes, sesquiterpenes, non-volatile fatty acids, and phenolic compounds—provide distinct chemical signatures that reflect genetic divergence (Deng *et al*., 2022). Gas chromatography-mass spectrometry (GC-MS) coupled with multivariate statistical techniques, such as hierarchical cluster analysis (HCA), enables the systematic categorization of taxa based on qualitative and quantitative chemical variations, offering objective criteria for species discrimination within Rutaceae.

Complementary to chemical profiling, palynology—the study of pollen grain morphology—provides highly stable, genetically conserved characters crucial for plant taxonomy. Pollen ultrastructure is largely independent of short-term environmental fluctuations, making it an ideal diagnostic feature for distinguishing closely related taxa at the genus and species levels (Abo-Elnaga *et al*., 2018; Garralla *et al*., 2021). The application of field-emission scanning electron microscopy (FESEM) allows high-resolution visualization of subtle micromorphological features, including exine sculpture (*e.g.*, reticulate, foveolate, or micro-reticulate patterns), aperture type and number, and precise polar and equatorial dimensions (Bilgin *et al*., 2022; Zhao *et al*., 2025). Integrating ultrastructural palynological data with phytochemical markers significantly enhances the reliability of biosystematic classification.

Despite the agricultural importance of *Citrus* in Iraq, comprehensive biosystematic studies integrating chemical profiling and ultrastructural palynology for local Iraqi germplasm are lacking. Most regional research has focused predominantly on basic agronomic performance, horticultural practices, or broad economic evaluations, leaving the precise taxonomic relationships and diagnostic markers of locally cultivated *Citrus* species poorly characterized. To address this critical research gap, the present study was designed to undertake an integrated biosystematics evaluation of eleven locally cultivated Citrus taxa collected from Diyala Province, Iraq. By synthesizing leaf metabolite profiles from GC-MS (analyzed via HCA) with high-resolution FESEM palynological observations, this study aims to: evaluate the diagnostic potential of chemical and pollen traits for species discrimination, establish clear taxonomic boundaries among the investigated taxa, and provide a robust scientific foundation for germplasm management and taxonomic resolution within the genus *Citrus* in Iraq.

Materials and Methods

Citrus samples

Healthy, mature leaf samples of eleven *Citrus* taxa were collected in December 2023 from agricultural research station orchards and private *Citrus* gardens across Diyala Province, Iraq. To account for potential intraspecific variation, samples were harvested from 3 to 5 individual mature, disease-free trees per taxon. Fully expanded, undamaged leaves were systematically collected from the middle portion of sunny shoots around the canopy of each tree. Taxonomic authentication of all collected taxa was performed based on standard morphological keys. Voucher specimens for all eleven studied *Citrus* species were prepared, properly processed, and deposited in the Herbarium of the University of Mazandaran (HUMZ), Babolsar, Iran.

The complete details regarding species identity, local names, collection coordinates, and assigned voucher specimen numbers deposited at HUMZ are comprehensively summarized in Table 1. For chemical extractions, leaf samples were thoroughly rinsed with distilled water to eliminate particulate matter and ambient contaminants, then shade-dried at room temperature (25 ºC) for two weeks. The dried tissues were homogenized into a fine powder using an electric grinder and stored in sealed, light-protected containers at 4 ºC until analysis. All extractions and subsequent analyses were performed in triplicate (n= 3 biological replicates per taxon).

Table 1. *Citrus* leaf samples were collected from evergreen *Citrus* gardens in Diyala

Population	Garden name	Lon	Lat	MA (m)	TP (mm)	MAT (°C)	HUMZ code
C. sinensis	Baquba	44°24'16.3″E	33°36'31.6″N	47	180	22.8	8012
C. aurantiumL	Dabbakha Farm	44°31'06.3″E	33°51'66110″N	55	190	22.5	8022
C. latifolia	Al-Musleh Farm	44°34'8545″E	33°61'9113″N	48	185	22.9	8014
C. sinensis var Moro	Diyala-New beach village	44°41'4750″E	33°62'7438 ″N	46	180	23.0	8015
C. deliciosa	Al-Abu Saida	44°77'0721″E	33°93'6637 ″N	62	200	22.5	8021
C. grandis	Al-Black village	44°57'08530″E	33°82'18290″N	65	195	22.6	8016
C. auarantiifolia var. acidica	Al-Jerusalam	44°35'3112″E	33°54'5450″N	58	195	22.7	8017
C. paradisi	Dali Abbas village	44°85'98110″E	34°07'59250″N	75	210	22.0	8018
C. limetta	Al-bakwba-hweder	44°64'2091″E	33°77'8491″N	52	190	22.8	8019
C. limon	Al-bakwba-hweder	44°64'2091″E	33°77'8491″N	52	190	22.8	8020
C. aurantium.	Al-Musleh Farm	44°34'8545″E	33°61'9113″N	48	180	22.9	8013

Lon=Longitude, Lat =Latitude, MA= Mean altitude, TP=Total precipitation per year, MAT=Mean annual temperate, HUMZ code= Herbarium of University of Mazandaran Code

Phytochemical extraction and GC-MS analysis

For leaf extract preparation, 2.0 g of finely powdered dried leaves were extracted with 20 mL of analytical-grade methanol (1:10 w/v solvent ratio) using ultrasonic-assisted extraction at room temperature (25± 2°C) for 45 minutes. The mixture was centrifuged at 4,000 rpm for 10 minutes, and the resulting supernatant was filtered through a 0.45 µm PTFE syringe filter before chromatographic analysis (Parobali *et al*., 2024).

GC-MS analysis was performed using an Agilent gas chromatograph coupled with a mass spectrometry detector. Compound separation was achieved using an HP-5MS capillary column (30 m length × 0.25 mm internal diameter, 0.25 µm film thickness). High-purity helium (99.999%) served as the carrier gas at a constant flow rate of 1.0 mL/min. The injector temperature was maintained at 250°C with a split ratio of 10:1. The oven temperature program was initialized at 50°C (held for 2 min), increased to 200°C at a rate of 5°C/min, and finally ramped to 280°C at 10°C/min (held for 5 min). The mass spectrometer was operated in electron impact ionization mode at 70 eV, with an ion source temperature of 230°C and a scan range of m/z 40- 550 amu. Phytochemical identification was established by comparing the obtained mass spectra with standard reference spectra in the National Institute of Standards and Technology (NIST) spectral library, accepting only compounds with a spectral similarity match index ≥ 85%.

Hierarchical cluster analysis

Hierarchical cluster analysis (HCA) was performed to evaluate the chemotaxonomic relationships among the eleven *Citrus* taxa based on their leaf GC-MS metabolite profiles. The input data matrix consisted of the relative percentage areas of identified chemical constituents across all samples. Prior to statistical modeling, data were normalized and log-transformed (log10 + 1) to reduce variance heterogeneity, followed by Z-score standardization (auto-scaling) to ensure equal weighting of both high-abundance and trace chemical compounds. The hierarchical dendrogram was constructed using Euclidean distance as the proximity metric and Ward's minimum variance method (Ward.D2) as the agglomerative clustering algorithm. To assess the statistical confidence and reliability of the identified clusters, multiscale bootstrap resampling was executed with 1,000 bootstrap iterations using R software (version 4.3.2) with the pvclust package. Cluster stability was evaluated using approximately unbiased (AU) p-values and bootstrap probability values, where clusters with AU≥ 95% were considered statistically strongly supported.

Palynological preparation and FESEM characterization

Pollen grains were harvested from mature, unopened flower buds of the eleven studied *Citrus* taxa. To remove surface lipophilic substances and prepare pollen samples for ultrastructural examination, pollen grains were processed following standard alcohol dehydration and air-drying protocols. Prepared pollen samples were mounted directly onto standard aluminum stubs using double-sided conductive carbon tape and sputter-coated with a 10 nm gold layer under high vacuum using an ion sputter coater. Micromorphological examination was performed using an FESEM (MIRA3TESCAN-XMU; Czech Republic) operated at a constant accelerating voltage of 15 kV. Micrographs were captured across a magnification range of 1500× (for whole grain habit and shape) to 20,000× (for fine exine ornamentation and micro-reticulation details). For quantitative palynological characterization, three randomly selected, fully developed, and non-collapsed pollen grains per species (sampled across 3 biological replicates per taxon) were measured using ImageJ software (v1.53k, National Institutes of Health, USA) spatially calibrated against the 10 µm scale bar of the micrographs. The quantified morphometric parameters included: polar axis length (P), equatorial diameter (E), P/E shape ratio, colpus length and width, exine thickness, and lumina/muri dimensions. All quantitative morphometric values were recorded as mean ± standard deviation (SD).

Data processing and artifact exclusion

To prevent solvent artifacts and laboratory contamination from confounding the phytochemical profiles, procedural blank samples (pure extraction-grade methanol processed identically through the extraction and filtration protocol without plant tissue) were analyzed under identical GC-MS conditions. Compounds identified in procedural blanks, including the extraction solvent peak (methanol) and trace aromatic artifacts (toluene and benzene), were systematically subtracted and excluded from the final qualitative and quantitative data matrix. Relative peak area percentages (%) were calculated strictly based on endogenous plant secondary metabolites.

Results

Chemotaxonomy analysis

GC-MS analysis of the methanolic leaf extracts of the eleven *Citrus* species revealed the presence of numerous volatile and semi-volatile compounds. The complete list of identified compounds, including retention time (RT) and relative peak area (%), is presented in Supplementary 1. For chemotaxonomic comparison, only compounds with a relative peak area greater than 1% were considered.

The detected compounds belonged mainly to hydrocarbons, fatty acids, alcohols and terpenoid-related compounds. Among them, compounds such as toluene, eicosane, henicosane, phytol and caryophyllene were detected in different relative abundances among the studied *Citrus* species. Minor compounds including citronellol and several fatty acid derivatives were also observed. It should be noted that some compounds such as toluene and benzene-related derivatives may originate from environmental contamination or analytical artifacts during sample handling or GC-MS analysis. Therefore, such compounds were not considered reliable chemotaxonomic markers in the comparative analysis.

Cluster analysis based on the chemical composition of the detected compounds in the methanolic leaf extracts of the eleven *Citrus* species was performed using XLSTAT software. The dendrogram obtained from this analysis is shown in Fig. 1. The results of the cluster analysis separated the studied *Citrus* species into two main groups. The first cluster (C1) included *C. sinensis*, *C. reshni*, *C. aurantium*, *C. latifolia*, *C. sinensis var.* Moro, *C. deliciosa*, *C. grandis*, *C. aurantiifolia var. acidica*, and *C. paradisi*. The second cluster (C2) consisted of two species, *C. limetta* and *C. limon*. This grouping reflects similarities and differences in the chemical composition of the detected compounds among the studied *Citrus* species.

Palynology analysis

Pollen grain measurements obtained from the studied *Citrus* species showed variation in several morphological characteristics, including polar axis (P), equatorial diameter (E), P/E, exine ornamentation, and pollen shape class (Table 2 and Fig. 2). Quantitative morphometric evaluation revealed marked variations in pollen dimensions among the studied *Citrus* taxa (Table 2). The P length ranged from 31.20 ± 1.50 µm in *C. deliciosa* to 41.20 ± 2.30 µm in *C. grandis*. Similarly, the E varied across species, with the lowest mean value recorded in *C. deliciosa*

(27.50 ± 1.20 µm) and the highest value observed in *C. grandis* (35.60 ± 1.80 µm). Overall, *C. grandis* exhibited the largest pollen dimensions, whereas *C. deliciosa* produced the smallest pollen grains among all examined species, as summarized in Table 2.

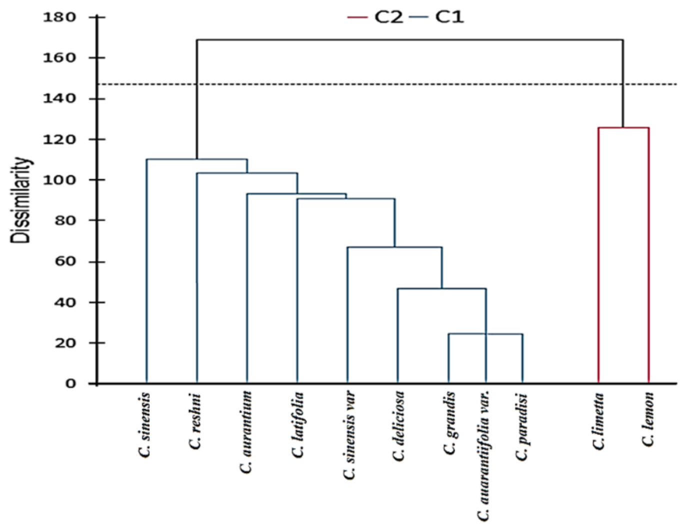

Fig. 1. A tree diagram from the chemical compounds among 11 *Citrus* species in Iraq. (C1: *C. sinensis*, *C. reshni*, *C. aurantium*, *C. latifolia*, *C. sinensis* var *Moro*, *C. deliciosa*, *C. grandis*, *C. auarantiifolia* var. *acidica*, and *C. paradise*; C2: *C. limetta*, *C. limon*).

Table 2. Pollen grain measurements of eleven *Citrus* species based on equatorial and polar views (mean ± SD, n= 3 pollen grains per species).

Species	P (µm)	E (µm)	P/E	Exine ornamentation	Pollen shape class
C. sinensis	34.15 ± 1.82	29.80 ± 1.45	1.15	Micro-foveolate / Punctate	Subprolate
C. latifolia	38.60 ± 2.10	32.40 ± 1.65	1.19	Punctate to foveolate	Subprolate
C. reshni	36.80 ± 1.95	33.10 ± 1.50	1.11	Densely punctate	Prolate-spheroidal
C. aurantium L.	35.40 ± 1.75	30.20 ± 1.30	1.17	Micro-foveolate	Subprolate
C. deliciosa	31.20 ± 1.50	27.50 ± 1.20	1.13	Finely punctate	Prolate-spheroidal
C. aurantiifolia var. acidica	33.50 ± 1.60	28.90 ± 1.35	1.16	Coarsely reticulate / Foveolate	Subprolate
C. grandis	41.20 ± 2.30	35.60 ± 1.80	1.16	Prominently reticulate	Subprolate
C. limon	32.80 ± 1.55	28.10 ± 1.25	1.17	Sparsely punctate	Subprolate
C. limetta	36.20 ± 1.85	31.00 ± 1.40	1.17	Micro-foveolate	Subprolate
C. paradisi	39.50 ± 2.15	34.20 ± 1.70	1.17	Punctate-foveolate	Subprolate
C. sinensis var. Moro	34.80 ± 1.70	30.10 ± 1.38	1.16	Finely punctate	Subprolate

P= Polar axis; E= Equatorial diameter.

In addition to axial measurements, the calculated polar-to-equatorial diameter ratio (P/E) provided essential diagnostic value for pollen shape classification among the studied *Citrus taxa* (Table 2). The P/E ratio ranged from 1.11 in *C. reshni* to 1.19 in *C. latifolia*. Based on standard palynological terminology, two distinct shape classes were recognized among the eleven taxa: prolate-spheroidal (P/E= 1.00- 1.14) and subprolate (P/E= 1.15- 1.33). The majority of the examined species—including *C. sinensis*, *C. latifolia*, *C. aurantium*, *C. aurantiifolia* var. acidica, C. grandis, *C. limon*, *C. limetta*, *C. paradisi*, and *C. sinensis* var. Moro—were characterized by subprolate pollen grains. Conversely, *C. reshni* (P/E= 1.11) and *C. deliciosa* (P/E= 1.13) exhibited prolate-spheroidal pollen grains, distinguishing them from the remaining taxa.

Discussion

Previous studies have reported a large diversity of phytochemical compounds in *Citrus* species. For example, Al-aamri *et al*. (2018) identified more than 200 chemical compounds in the methanolic extract of *Citrus* leaves. In the present study, several major compounds were detected in the analyzed *Citrus* species using GC-MS analysis. The most prominent compounds included toluene, benzene-methanol, n-hexadecanoic acid, pentacosanoic acid methyl ester, nonacosane, neophytadiene, henicosane, oleic acid, linalyl acetate, phenol derivatives, and myristic acid. Although the diversity of compounds identified in the present study was lower than that reported by Al-aamri *et al.* (2018), the detected compounds represent important phytochemical constituents commonly reported in *Citrus* species. The relative concentrations of these compounds varied among the studied species according to the GC-MS results obtained in this study, indicating chemical variability within the genus *Citrus* .

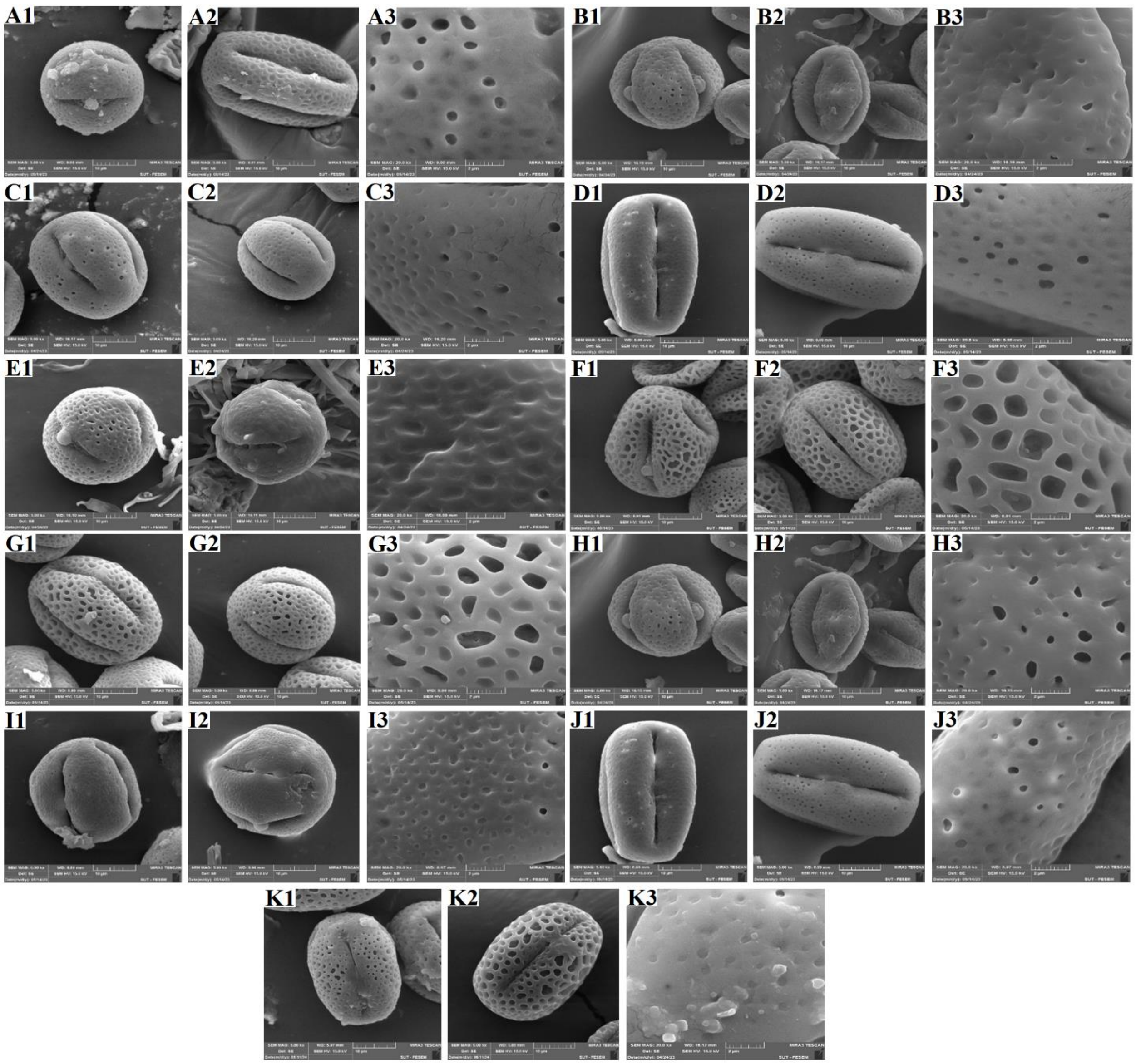

Fig. 2. FESEM images of pollen grains from *Citrus* species in Iraq: A1-3) *C. auarantiifolia var. acidica*; B1-3) *C. aurantium*; C1-3) *C. deliciosa*; D1-3) *C. grandis*; E1-3) *C. latifolia*; F1-3) *C. limetta*; G1-3) *C. limon*; H1-3) *C. sinensis*; I1-3) *C. sinensis var.* Moro; J1-3) *C. paradise*; and K1-3) *C. reshni*; Alphabet subscripted to numbers of 1-3 indicated that Equatorial with 10 µm size, Polar with 10 µm size, and Pollen grain membrane with 2 µm, respectively.

Several studies have shown that *Citrus* leaves contain large amounts of chemical compounds such as toluene, methylbenzene, hexane, eicosane, oleic acid, and others, in varying proportions. In fact, Deng *et al*. (2022) characterized the chemical composition and functional properties of *Citrus* leaf extracts from *Citrus sinensis* (Uzbek), *Citrus aurantium and C. sinensis var Moro*. Their results indicated that the main chemical compounds were limonene, β-pinene, sabinene, β-ocymine, β-copene, terpinen-4-ol, and linalool. *Citrus* leaves are characterized by high levels of limonene, α-phellandrene, β-caryophyllene, nerol, and citronellol (Deng *et al.*, 2022). Another study by Khan *et al*. (2018) showed that *Citrus* leaves are a rich source of important phytochemicals, yielding results similar to those obtained previously. This discovery was achieved through the analysis of *Citrus* leaf extracts using GC-MS, which revealed the presence of monoterpene hydrocarbons, alcohols, sesquiterpene hydrocarbons, aldehydes, monoterpenoids, esters, beta-caryophyllene, beta-ellimine, acetates, and other chemical compounds. These techniques represent an important classification method for determining the homogeneity or heterogeneity of citrus species (Khan *et al.*, 2018). Furthermore, Anmol *et al*., (2021) demonstrated that *Citrus* (Rutaceae) leaves exhibit diverse biological activities of their extracted chemical compounds, including flavonoids, polyphenols, coumarins, limonoids, acridon alkaloids, essential oils, vitamins, and others. Our study yielded similar results, including terpenes, phenols, and fatty acids, which we obtained using GC-MS. These compounds possess a high antioxidant effect (Anmol *et al.*, 2021). Furthermore, Rao *et al.*, (2023) conducted a study highlighting the importance of *Citrus* fruits due to their high content of important flavonoid compounds. These compounds were found in varying proportions and concentrations in both the leaves and fruits. The study concluded that the dried leaves contain high levels of flavonoids, such as vitexin, genistein, kaempferol, 3-neohesperidoside, eriocarpin, luteolin, and flavanols. These compounds were compared to those found in species used in our study, including dihydrocoumarin, phenol, 2,4-Di(1,1-dimethylethyl), 2,4-di-tert-butylphenol, 5-phenyl-4,5-dihydro-1H- [1,2,4], benzene-methanol, phenol, and 5-methyl-2-(1-methylethyl). The results revealed a clear diversity of these compounds, which have been used as antioxidants, anti-inflammatories, and anticancer agents, thus contributing to the development and enhancement of the body's immunity (Rao *et al.*, 2023). Research conducted by Posadino *et al.*, (2024) showed the presence of flavonoids, terpenes, and other compounds with different concentrations. It also noted the presence of other compounds such as limonene, myrcene, and various oxidized monoterpenes in different proportions that are extracted from the peels and leaves of *Citrus* fruits. In our study of several *Citrus* species, we obtained similar results for chemical compounds, including flavonoids, terpenes, esters, fatty acids, alkanes, and other compounds of high taxonomic value, which can be used to determine the important taxonomic features of *Citrus* species (Posadino *et al.*, 2024). However, a study by Saidi *et al.*, showed that essential oil compounds, flavonoids, alpha-pinene, tannins, phenolic acid, and others offer a variety of health benefits thanks to their powerful antioxidant and anti-inflammatory properties. This was also observed in our results from methanolic extracts of *Citrus* leaves, which showed varying concentrations of compounds such as 2,4-Di-tert-butylphenol (0.33%) in *Citrus (C. limon*), 2-pentanone, 4-hydroxy-4-methyl- (1.55%) in *Citrus sinensis*, docosane (17.86%) in *Citrus (C. latifolia*), 3,2-hexadecine-1-ol, 3,7,11,15. tetra (0.45%) in *Citrus (C. grandis*), hexadecanoic acid, methyl ester (1.22%) in *C. deliciosa*, and dihydrocoumarin, 4,4,5,7,8-penta (5.06%) in *C. aurantium*, among others, which were present in varying proportions among the different species (Saidi *et al.*, 2022). *Citrus* leaves have recently garnered significant attention due to their antioxidant properties. A study by Zhao *et al.*, (2025) indicates that these chemical compounds, particularly fatty acids, which are abundant in the leaves and fruits, play a crucial role in their interactions with the environment. We obtained high levels of fatty acids, noting that the type was particularly prevalent. *C. paradisi* contains oleic acid (12.84%) and octadecanoic acid (5.79%), while *C. reshni* was also found to be abundant in hexadecenoic acid (1.27%) and hexadecenoic acid (1.27%). *C. sinensis* also contains n-hexadecanoic acid (4.58%) and methyl 2-methoxyoctadecanoate (1.94%). *C. limon* contains the fatty acid E-9-tetradecenoic acid (0.32%), while *C. limetta* contains n-hexadecanoic acid (2.58%) and octadecanoic acid (0.87%). *C. deliciosa* contains n-hexadecenoic acid (0.98%) (Zhao *et al.*, 2024). Another study revealed that essential oils extracted from *Citrus* leaves, such as orange, lemon, and grapefruit, possess a distinctive aromatic scent. Results were obtained for essential oils from several species, including *C. aurantium*, which contains neovitadine and dihydrocoumarin; *C. grandis*, which contains neovitadine; *C. latifolia*, which contains both neovitadine and a diterpene; *C. limetta*, which contains caryophyllene and bicyclo-ondesicin; *C. limon*, which contains linalool, linalyl acetate, caryophyllene, and 2,4-Di-tert-butylphenol; *C. sinensis*, which contains the essential oils cycloisolongivolin-8-oxo and allyl cyclohexyl carbonate, and *C. reshni*, which contains many aromatic compounds, including linalool, dimethyl octadinol, and methylphenol, is a species that includes *C. deliciosa*, *C. paradisi*, and *C. grandis*, which are very poor in aromatic compounds (Rana *et al.*, 2025). In the same context, Elhawary et al. pointed to the importance of essential oils found in the leaves, peels, and flowers of *Citrus* fruits, which support plant growth and development. We obtained varying results regarding the presence of essential oils among different species. For example, *C. limon* showed linalyl acetate (0.35%) and 2,4-Di-tert-butylphenol (0.33%). *C. reshni* contained only L-Linalool (1.60%), phenol, 5-methyl-2-(1-methylethyl) (6.05%), and 9-Octadecenoic acid, methyl ester (3.04%). *C. limetta* contained only Bicycle [2,2,1] heptane, 2-methyl (0.16%). *C. sinensis* contained a lower percentage of volatile oils than the other species, specifically Allylcychohexyl carbonate (5.79%) (Elhawary *et al.*, 2024). In a study conducted by Owolabi *et al*., (2018) it was found that *Citrus* leaves, fruits, seeds, stems, and peels contain a wide range of phytochemicals with potent effects. Significant and diverse results were obtained from compounds such as carbohydrates, proteins, alkaloids, essential oils, flavonoids, saponins, steroids, terpenoids, glycosides, and others, using GCMS technology. This analysis allowed for the identification of the chemical properties of these compounds, enabling the classification of these species (Owolabi *et al.*, 2018).

Our results showed that pollen grains in all studied varieties were unicellular, radially symmetrical, and isopolar. The pollen grains were generally medium-sized, with the exception of C. *paradisi*, *C. reshni, and C. trifoliate*. which ranged in size from small to medium. Their shapes were semi-elongated, spherical, flattened, and spherical (Lora *et al.*, 2022). The diversity of taxonomic features within the *Citrus* genes itself makes it more complex both taxonomically and morphologically. Following Dutra and Gasparino (2018) who used SEM techniques to analyz variations in pollen grains size, results showed differences in pollen grains size among the studied species. Some *Citrus* species, such as C. deliciosa, exhibit a high area (Equatorial) value (0.054) compared to others, such as C. sinensis (0.032). This indicates variation in pollen grain size (Dutra and Gasparino, 2018). The result of one study showed variation in pollen grains shapes, including spheroidal, sub - prolate, and oblate. Elongated pollen grains were observed in the genus *C. paradisi*, while spherical pollen grains were found in *C. sinensis var.* Moro, *C. sinensis, C. limon, and C. latifolia.* These characteristics helped to distinguishing the morphology of the studied *Citrus* species (Al-Anbari *et al.*, 2015). The study demonstrates that when external carving varies between the studied species, observed in *(C. aurantium)* of *C. deliciosa and C. grandis*, present study confirms that the pollen grain wall in *C. aurantium* belongs to the same group (Bilgin *et al.*, 2022). Ding *et al.*, (2024) used scanning electron microscopy to characterize the morphological properties of pollen grains, focusing on the horizontal and vertical axes. In the current study, differences in polarity values between species were observed. Some species, such as *C. reshni, C. aurantium, and C. grandis*, exhibited very high polarity, while others, such as *C. deliciosa*, showed lower terminal polarity (Ding *et al.*, 2024). Dutra and Gasparino (2018) revealed variation in pollen grain morphology, with a large proportion being oval, followed by round or oblong. The size and shape of the pollen grains were examined by using SEM. They found flattened or spherical shapes in *C. deliciosa, C. paradisi, C. trifolia, and C. aurantium*; and nearly spherical shape in *C. reshni, C. sinensis var.* Moro, *C. sinensis, C. limon, C. limetta, C. grandis and C. aurantiifolia var. acidica* (Dutra and Gasparino, 2018). While the hierarchical clustering based on combined palynological and GC-MS data provided distinct grouping patterns among the eleven Citrus taxa, these results should be interpreted as phenetic similarities rather than definitive phylogenetic relationships. Chemotaxonomic markers and micromorphological characters offer valuable diagnostic support for species discrimination; however, chemical convergence and environmental plasticity can influence metabolic profiles. Therefore, while our multivariate findings effectively clarify the degree of phytochemical and palynological similarity among these taxa, comprehensive molecular phylogenetic analyses (*e.g.*, plastid DNA sequencing or nuclear markers) remain indispensable to fully resolve the evolutionary lineage and taxonomic framework of the genus Citrus.

Conclusion

The present study demonstrates the taxonomic significance of chemical and palynological characteristics in the differentiation of *Citrus* species in Iraq. The GC-MS analysis of leaf methanolic extracts revealed variations in the chemical composition among the studied species, which provided useful chemotaxonomic markers for their classification. Hierarchical Cluster Analysis (HCA) based on the detected chemical compounds separated the studied *Citrus* taxa into two main clusters. The first cluster (C1) included *C. sinensis*, *C. reshni*, *C. aurantium*, *C. latifo*lia, *C. sinensis var.* Moro, *C. deliciosa*, *C. grandis*, *C. aurantiifolia var. acidica*, and *C. paradisi*, whereas the second cluster (C2) consisted of *C. limetta* and *C. limon*. In addition, the pollen grain characteristics by FESEM of the studied *Citrus* species revealed biodiversity and taxonomic characters within the *Citrus* genus. The results also revealed significant variations in pollen grain characteristics among the studied species, reflecting the considerable taxonomic diversity in this genus. Together, the chemotaxonomic and pollen grain characteristics were important taxonomic features that contribute to differentiating and distinguishing among different species of the *Citrus* genus.

Conflict of interests

The authors have no conflicts of interest to declare.

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